MedChemComm

CONCISE ARTICLE

View Article Online
View Journal | View Issue

Cite this: *Med. Chem. Commun.*, 2014, **5**, 468

Synthesis and biological evaluation of new conformationally restricted *S*-DABO hybrids as non-nucleoside inhibitors of HIV-1 reverse transcriptase†

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Received 31st August 2013
Accepted 4th January 2014

DOI: 10.1039/c3md00247k

www.rsc.org/medchemcomm

A series of conformationally restricted dihydro-alkylthio-benzyl-oxopyrimidine (*S*-DABO) hybrids, which combined the structural features of C6-α-methylbenzyl-thio-DABOs (α-methyl-*S*-DABOs) and C6-α-cyanobenzyl-thio-DABOs (CN-*S*-DABOs), has been synthesized and biologically evaluated for their anti-HIV activity against wild-type HIV-1 strain IIIB, double RT mutant (K103N + Y181C) strain RES056 and HIV-2 strain ROD in MT-4 cell cultures. Most of these compounds exhibited inhibitory activity (wild-type) within the range of EC_{50} values from micromolar to nanomolar. Among them, compound **1s** displayed the highest anti-HIV-1 activity with an EC_{50} value of 91 nM and a selectivity index (SI) of 548, which was more potent than zalcitabine and comparable to nevirapine and delavirdine in the same assay. The HIV-1 reverse transcriptase inhibitory (RT) assay confirmed that these conformationally restricted *S*-DABO hybrids targeted HIV-1 RT. The preliminary structure–activity relationship (SAR) and molecular docking analysis of this new series of conformationally constrained CN-*S*-DABO hybrids were also investigated.

Introduction

Non-nucleoside reverse transcriptase inhibitors (NNRTIs), known as one of the indispensable components of highly active antiretroviral therapy (HAART), have attracted wide attention due to their high specificity, excellent potency and low cytotoxicity.[1–3] With years of efforts, many kinds of NNRTIs with diverse structures have been developed, such as dihydroalkoxybenzyloxopyrimidines (DABOs), 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymines (HEPTs), diaryltriazines (DATAs), and diarylpyrimidines (DAPYs).[3–5]

Since the first series of DABOs were disclosed as a novel class of NNRTIs in 1992,[6] many structural modifications have been made and led to the discovery of numerous promising DABO families such as *S*-DABOs, *N*-DABOs, DATONs, DABOCs and α-methyl-DABOs (Fig. 1).[4,7,8] Among them, conformationally restricted α-methyl-*S*-DABOs characterized by a methyl group at the methylene bridge between the pyrimidine and phenyl rings exhibited excellent inhibitory potencies against both wild-type HIV-1 and drug-resistant mutants without significant cytotoxicity at higher concentrations.[9–18] Upon analysis of the left wing of α-methyl-*S*-DABOs, the *p*-methoxybenzyl moiety seems to be the most favorable for the interactions with the entrance of the non-nucleoside inhibitor binding pocket (NNIBP).[14]

In our previous DABO project,[19] we introduced a CN group at the methylene bridge and obtained a novel series of potent CN-*S*-DABOs. The most active compound **3** (Fig. 2) showed strong inhibitory potency with an EC_{50} value of 2 nM. The docking study revealed that its α-cyanobenzyl moiety fitted into the aromatic-rich NNIBP, surrounded by the aromatic side chains of Tyr181, Tyr188, Phe227 and Trp229, as well as Leu234 and Pro95. Considering that the α-CN group at the benzylic position could enhance π–π stacking interaction with the surrounding aromatic residues and it could also result in a restricted conformation as **2**, we combined the structural features of **2** and **3** to generate a new series of CN-*S*-DABO hybrids with the aim to develop a novel class of conformationally restricted *S*-DABO analogs in present work. Herein we report the synthesis and biological evaluation of these new α-CN-*S*-DABO hybrids **1** (Fig. 2).

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† Electronic supplementary information (ESI) available: Includes the detailed experimental procedures and analytical data for all new compounds, materials and methods for biological evaluation and molecular simulation. See DOI: 10.1039/c3md00247k

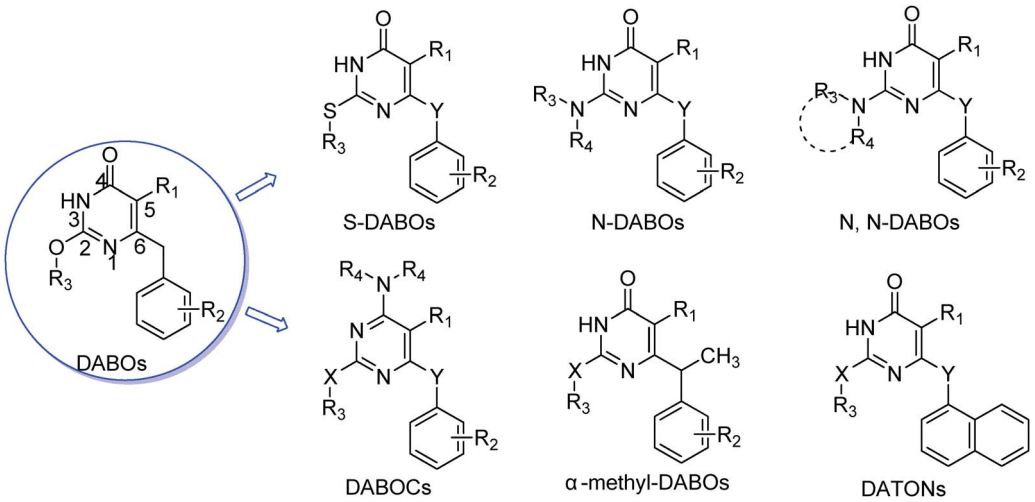


Fig. 1 Typical representations of the DABO family.

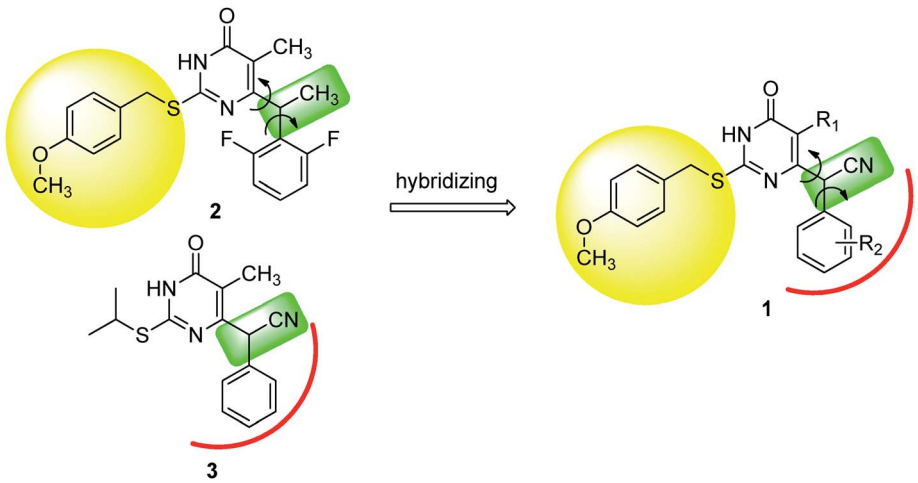


Fig. 2 The lead compounds and newly designed C6-rigid *S*-DABOs **1**.

Results and discussions

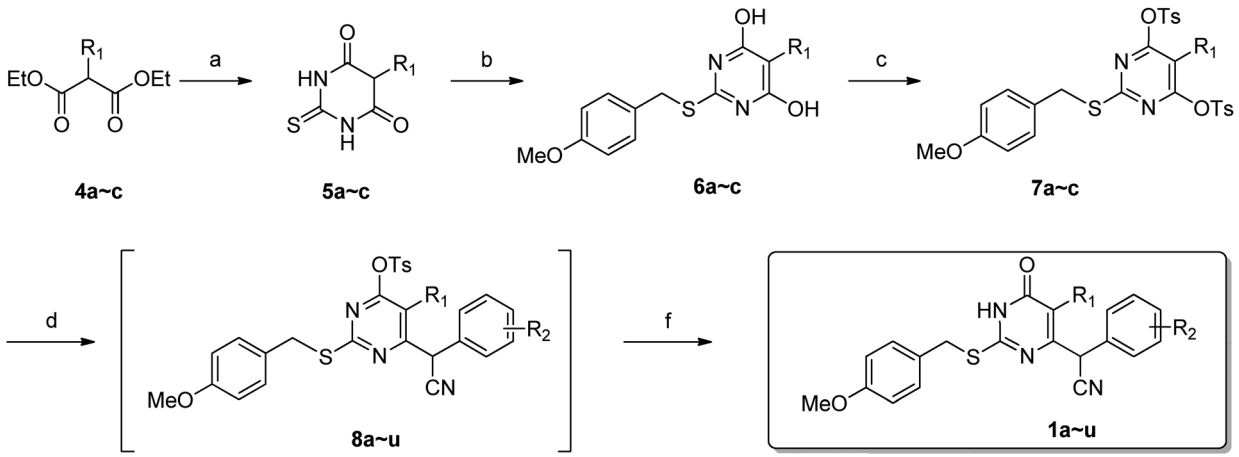
Chemistry

The newly designed compounds **1a–u** were obtained from common intermediates **7a–c**, which were easily synthesized in three steps, as depicted in Scheme 1. The 2-thiobarbituric acid derivatives **5a–c** were prepared according to the method of Koroniak *et al.*[20] by condensation of thiourea with the corresponding diethyl malonates **4a–c** using sodium methoxide as base in methanol under refluxing conditions. Subsequently, **5a–c** were *S*-alkylated with freshly made 4-methoxybenzyl chloride,[21] producing the corresponding 5-alkyl-2-alkylthiobarbituric acids **6a–c** which were then treated with *p*-toluenesulfonyl chloride and K_2CO_3 in anhydrous DMF to afford disulfonates **7a–c**. These common intermediates were then reacted with various substituted benzyl cyanide in the presence of 60% NaH and air in anhydrous DMF to yield **8a–u**, which were hydrolyzed with 30% aqueous sodium hydroxide to obtain the target compounds **1a–u**.

Biological evaluation

Anti-HIV activity evaluation on cell cultures. The new series of α-CN-*S*-DABO hybrids was biologically evaluated for their anti-HIV activity against wild-type HIV-1 strain IIIB, double RT mutant (K103N + Y181C) strain RES056 and HIV-2 strain ROD in MT-4 cell cultures, in parallel with nevirapine (NVP), zidovudine (AZT), zalcitabine (DDC), efavirenz (EFV) and delavirdine (DLV) as reference drugs in the test. The results, expressed as EC_{50} (anti-HIV activity), CC_{50} (cytotoxicity) and SI (selectivity index, given by the CC_{50}/EC_{50} ratio), are summarized in Table 1.

As seen from the results listed in Table 1, most of these CN-*S*-DABOs (**1a**, **1c–d**, **1f–j**, **1l–q**, **1s–u**) showed moderate to good inhibitory activity against wild-type HIV-1 with EC_{50} values ranging from 29.08 to 0.09 μM. Among them, compound **1s** displayed the highest potency with an EC_{50} value of 0.09 μM and a selectivity index (SI) > 548, which was more potent than DDC (EC_{50} = 0.57 μM, SI = 169), comparable to NVP (EC_{50} = 0.11 μM, SI = 132) and DLV (EC_{50} = 0.11 μM, SI = 338), but more selective than these reference drugs.



Scheme 1 Synthesis of compounds **1a–u**. *Reagents and conditions*: (a) thiourea, MeONa, MeOH, reflux, 4–8 h; (b) 4-methoxybenzyl chloride, NaOH, H_2O, 40 °C, 4–6 h; (c) TsCl, K_2CO_3, DMF, rt., 8–12 h; (d) substituted benzyl cyanide, 60% NaH, DMF, −5 °C to rt., 12–24 h; (e) NaH, DMF, air, rt., 12–24 h; (f) 30% NaOH, 6–8 h.

Preliminary SAR information can be summarized from the data in Table 1 as following: (a) variation in substitution at the C-5 position of the pyrimidine core had influence on antiviral potency. Interestingly, the effect of substitution at the C-5 position was greatly influenced by the choice of the C-6 benzylic substituent. Compound **1p** (5-iPr, EC_{50} = 5.72 μM) exhibited higher potency than that of **1g** (5-Me, EC_{50} = 16.28 μM), with a bromide atom at the *meta* position of the C-6 α-cyanobenzyl

Table 1 Anti-HIV-1 activities and cytotoxicity of compounds **1a–u** in MT-4 cells[a]

			EC_{50}[b] [μM]				
Compd	R_1	R_2	WT (IIIB)	K103N + Y181C	HIV-2	CC_{50}[c] [μM]	SI[d]
1a	Me	3-F	29.08 ± 2.85	>172.79	>172.79	172.79 ± 25.01	6
1b	Me	4-F	>95.17	ND[e]	>95.17	95.17 ± 72.46	1
1c	Me	2-Cl	0.49 ± 0.32	>115.51	>115.51	115.51 ± 9.14	239
1d	Me	3-Cl	18.00 ± 2.27	>160.72	>160.72	160.72 ± 18.09	9
1e	Me	4-Cl	>28.90	ND	>28.89	28.89 ± 1.95	1
1f	Me	2-Br	0.42 ± 0.15	>86.65	>86.65	86.65 ± 12.84	208
1g	Me	3-Br	16.28 ± 0.55	>155.91	>155.91	155.91 ± 12.75	10
1h	Et	2-F	1.66 ± 0.44	>108.77	>108.77	108.77 ± 57.32	66
1i	Et	3-F	7.08 ± 1.14	>172.75	>172.75	172.75 ± 19.60	24
1j	Et	2-Cl	1.34 ± 0.46	>76.87	>76.87	76.87 ± 40.90	57
1k	Et	4-Br	>23.87	ND	>23.87	23.87 ± 0.97	1
1l	i-Pr	4-F	20.45 ± 10.01	>136.67	>136.67	136.67 ± 25.29	7
1m	i-Pr	2-Cl	3.91 ± 0.24	>27.36	>27.36	27.36 ± 2.59	7
1n	i-Pr	4-Cl	>40.68	>85.18	>85.18	85.18 ± 42.04	≤2
1o	i-Pr	2-Br	4.46 ± 1.33	>26.67	>26.67	26.67 ± 2.75	6
1p	i-Pr	3-Br	5.72 ± 1.00	>118.66	>118.66	118.66 ± 17.62	21
1q	Me	2,6-diCl	0.19 ± 0.06	>48.80	>48.80	48.80 ± 33.55	252
1r	Me	2-F-4-Br	>22.52	ND	>22.52	22.52 ± 0.93	1
1s	Me	2-F-6-Cl	0.091 ± 0.027	>50.10	>50.10	50.10 ± 26.55	548
1t	Me	2,4-diF	20.66 ± 4.07	>156.89	>156.89	156.89 ± 22.38	8
1u	Me	3,5-diF	21.31 ± 2.27	>153.56	>153.56	153.56 ± 14.33	7
NVP			0.11 ± 0.07	5.12 ± 5.14	ND	>15.02	>132
AZT			0.0064 ± 0.0024	0.0063 ± 0.00003	0.0060 ± 0.0010	>93.56	>14 445
DDC			0.57 ± 0.14	ND	1.61 ± 1.02	>94.70	>169
EFV			0.0089 ± 0.0015	0.51 ± 0.14	ND	>6.34	>727
DLV			0.11 ± 0.07	>36.19	ND	>36.19	>338
2 (ref. 14)			0.000025	ND	ND	>12.43	
3 (ref. 19)			0.002 ± 0.0002	ND	ND	10.81 ± 6.56	4600

[a] Data represent the mean of at least three separate experiments. [b] Compound concentration required to protect MT-4 cells against viral cytopathogenicity by 50%. [c] Compound concentration that decreases the uninfected MT-4 cell viability by 50%. [d] Selectivity index: CC_{50}/EC_{50} (WT) ratio. [e] ND: not determined.

ring. Similarly, compound **1l** (5-iPr, $EC_{50} = 20.45$ μM) was more potent than **1b** (5-Me, $EC_{50} > 95.17$ μM), with a fluoro atom at the *para* position. In addition, compound **1i** (5-Et, $EC_{50} = 7.08$ μM) with a fluoro atom at the *meta* position also displayed higher antiviral activity than that of **1a** (5-Me, $EC_{50} = 29.08$ μM). It seems that a bulky group is favourable for improving inhibitory activity when there is a substituent at the *meta* or *para* position of the C-6 α-cyanobenzyl ring, which is consistent with the previous reported findings.[19] However, for the compounds with a substituent at the *ortho* position, the inhibitory activity decreased in the order: Me (**1c**) > Et (**1j**) > iPr (**1m**). (b) Despite the substituent at the C-5 position of the pyrimidine core, introduction of a substituent at the *para* position of the C-6 α-cyanobenzyl ring is not tolerable. All the compounds (**1b**, **1e**, **1k**, **1l**, **1n**) with a substituent at the *para* position completely lost potency against HIV-1 replication, except for **1l**. Introduction of a substituent at the *ortho* position is favourable, compounds **1c**, **1f**, **1h** and **1o** proved to be more potent than the corresponding *meta* position substituted compounds **1d**, **1g**, **1i** and **1p**, respectively. In addition, introduction of an additional substituent at the *ortho* position led to significant improvement in inhibitory potency. Compound **1q** (2, 6-diCl, $EC_{50} = 0.19$ μM) and **1s** (2-F-6-Cl) were more potent than **1c** (2-Cl, $EC_{50} = 0.49$ μM).

HIV-1 RT inhibition assay

To confirm that RT is the biological target of these newly synthesized α-CN-*S*-DABO hybrids, four representative compounds were selected to carry out an HIV-1 RT inhibitory assay according to the method described by the manufacturer of the EnzCheck RT Assay kit (Molecular Probes, Invitrogen); nevirapine, efavirenz (EFV) and etravirine (TMC-125) were also tested in the same assay as the reference drugs.

As shown in Table 2, all the tested compounds showed potent inhibitory activity against HIV-1 RT and were more potent than NVP. This strongly supports that the synthesized hybrids in this study act as NNRTIs. Interestingly, these four compounds have higher inhibitory activity in HIV-1 replication (Table 1) than in the RT assay. The reason for this phenomenon might be that those compounds also inhibit DNA-dependent DNA polymerase activity, which could not be optimally detected by the RT assay used in this study.

Molecular simulation

To investigate the potential binding mode of the newly synthesized compounds with HIV-1 RT, molecular simulation was conducted by using Sybyl Surflex-Dock program, and the crystallographic structure of wild type RT in complex with TNK-651 (PDB ID: 1RT2) was used due to the high degree of similarity between TNK-651 and *S*-DABOs. The most active compound **1s** either on cell cultures or in RT assay was taken as a representative compound and chosen for docking analysis. The modelling method is illustrated in detail in the ESI,† and theoretical binding mode of **1s** in the NNIBP is shown in Fig. 3. To test the accuracy and reliability of the docking procedure, the reference compound TNK-651 taken from the crystal complex was docked into the NNIBP to predict the binding geometries, which successfully reproduced the experimental geometries well with an acceptable root-mean-square deviation (RMSD) value of 0.527 Å (see Fig. 4).

The docking simulation clearly shows that **1s** forms two hydrogen bonds with the residue Lys101 of HIV-1 RT. One was observed between the NH of the pyrimidinone ring and the peptidic carbonyl oxygen of Lys101, another was between the C=O fragment of the pyrimidinone ring and the NH of Lys101, with atom-pair distances of 1.81 Å and 2.13 Å, respectively. This might efficiently stabilize the NHC=O fragment at the N3/C4 positions of the pyrimidinone ring and lower the energy of the active conformations of **1s** in NNIBP. Therefore, these two hydrogen bonds play an important role in the strong potency of **1s** against HIV-1 RT. The methyl group at the C5 position and the substituted α-cyanobenzyl moiety of **1s** at the C6 position were located in an aromatic rich hydrophobic region defined by the aromatic side chains of Tyr181, Tyr188, Trp229, as well as Tyr 232 and Pro95. Notably, the electron-deficient 2-F-6-Cl-benzyl moiety was almost parallel with the electron-rich benzene ring of Tyr181 residue, which would result in a strong

Table 2 Anti-HIV-1 RT (WT) activity of representative compounds[a]

Compd	1c	1f	1q	1s	NVP	EFV	TMC-125
IC_{50}[b] [μM]	0.51	1.55	0.23	0.17	1.78	0.037	0.017
SD[c]	0.12	0.51	0.05	0.04	0.19	0.019	0.006

[a] Data represent the mean of two separate experiments. [b] Compound concentration required to inhibit HIV-1 RT (WT) activity by 50%. [c] Standard deviation.

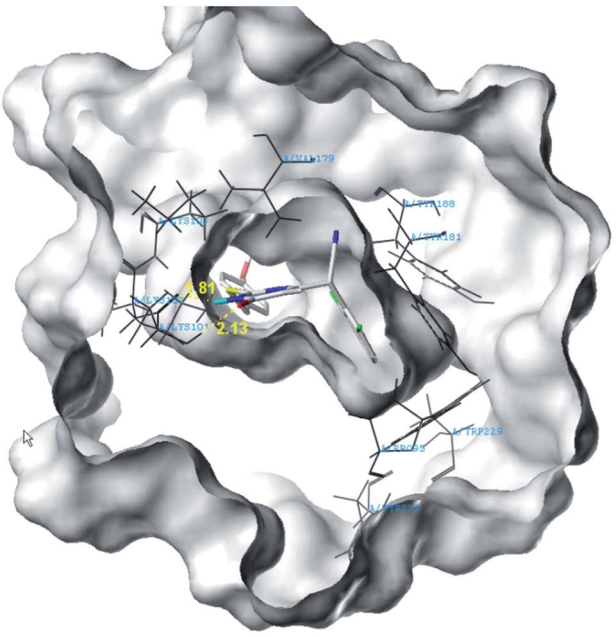


Fig. 3 Predicted binding mode of compound **1s** and the non-nucleoside inhibitor binding pocket (NNIBP) of wild-type HIV-1 RT (PDB code: 1RT2). Hydrogen bonds are indicated with dashed lines in yellow.

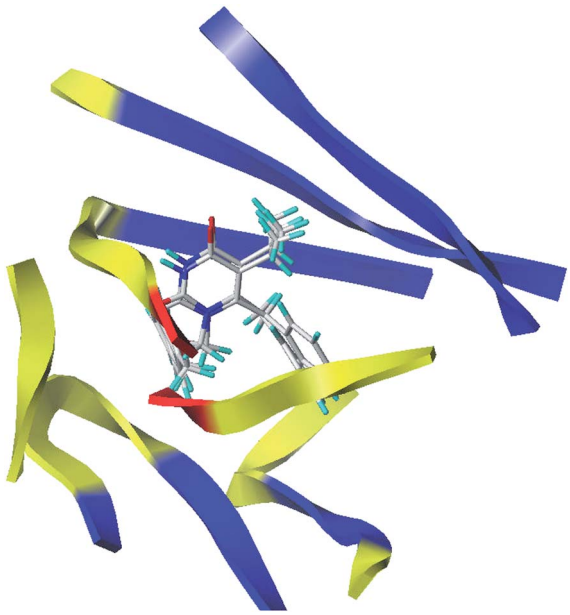


Fig. 4 Comparison of the docked TNK-651 and X-ray conformation of TNK-651 into NNIBP.

π–π stacking interaction. The cyano group linked to the benzyl ring further enhanced this π–π stacking interaction. Unfortunately, the orientation of the *p*-methoxybenzyl moiety at the C2 position was not maintained, directing to residues Val179 rather than Asp192, and pushing the residue Val179 and Tyr181 away from Tyr188, which might disrupt the entrance of the binding pocket and the hydrogen bond network between RT residues, which played an important role in the excellent activity of **6a**.[14]

Conclusion

In summary, a series of new conformationally restricted *S*-DABO hybrids with a substituted α-cyanobenzyl group at C6 of pyrimidine were synthesized and evaluated in cellular assays. Most of the target compounds showed inhibitory activity against wild-type HIV-1 with EC_{50} values ranging from 40.68 to 0.09 μM. The most potent compound **1s** exhibited a low EC_{50} value of 0.09 μM and SI > 548, and was more potent than the reference drug DDC and comparable to NVP and DLV, but more selective. The HIV-1 RT assay confirmed that these newly synthesized compounds targeted HIV-1 RT. The present biological and docking studies revealed some important SARs for this series of *S*-DABO hybrids: (a) the pyrimidine core is crucial for maintaining inhibitory potency by forming H-bonds with Lys101, (b) introduction of electron deficient substituent at the *ortho* position of the C-6 benzyl ring is helpful for enhancing affinity of the inhibitors and RT, (c) a small group is favorable for improving inhibitory potency when substituted at the *ortho* position of the C-6 benzyl ring, (d) incorporation of a CN group at the C-6 benzyl ring can enhance π–π stacking interaction with Tyr181, Tyr188, Trp229, Tyr232 and Pro95. Further structural optimization of *S*-DABOs analogues will take into account these aspects for developing more active and selective NNRTIs.

Acknowledgements

This research was financially supported by grants from National Natural Science Foundation of China (no. 81172918), Shanghai Municipal Natural Science Foundation (no. 13ZR1402200), Chinese National Science and Technology Major Project (no. 2012ZX09103101-068) and KU Leuven (GOA 10/014).

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